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An Essential Role for Retinoid Receptors RARβ and RXRγ In Long-Term Potentiation and Depression

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Summary

Hippocampal long-term potentiation (LTP) and long-term depression (LTD) are the most widely studied forms of synaptic plasticity thought to underlie spatial learning and memory. We report here that RARβ deficiency in mice virtually eliminates hippocampal CA1 LTP and LTD. It also results in substantial performance deficits in spatial learning and memory tasks. Surprisingly, RXRγ null mice exhibit a distinct phenotype in which LTD is lost whereas LTP is normal. Thus, while retinoid receptors contribute to both LTP and LTD, they do so in different ways. These findings not only genetically uncouple LTP and LTD but also reveal a novel and unexpected role for vitamin A in higher cognitive functions.

Introduction

Vitamin A and its derivatives (the retinoids) participate in many essential physiological processes, including vision, reproduction, epithelial differentiation, hematopoiesis, bone development, and pattern formation during embryogenesis (Sporn et al., 1994). Vitamin A deficiency (VAD) is most common in children and dramatically increases in poor and undernourished populations. Severe VAD results in blindness and is associated with 200,000 deaths per year (Sommer, 1982).

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Retinoid signaling is mediated by two classes of receptors, retinoic acid receptors (RAR α, β, and γ) and retinoid X receptors (RXR α, β, and γ) (Mangelsdorf et al., 1994). These receptors belong to the steroid/thyroid hormone nuclear receptor superfamily and function as transcription factors by binding to retinoid response elements in the promoters of target genes predominantly in the form of RAR/RXR heterodimers (Chambon, 1994; Mangelsdorf et al., 1994; Kastner et al., 1997). The multiplicity of receptor subtypes in each family suggests that different heterodimers may be involved in distinct physiological processes. Homologous recombination studies have established a clear link between VAD and the receptors (Kastner et al., 1997; Sucov et al., 1994 and references therein).

All of the subtypes of RAR and RXR are different gene products with each displaying distinct patterns of expression (Mangelsdorf et al., 1994). RARα is almost ubiquitously expressed (Giguère et al., 1987; Dollè et al., 1990; Ruberte et al., 1991), whereas the RARβ and γ genes display more restricted expression patterns (Dollè et al., 1990; Ruberte et al., 1993; Mangelsdorf et al., 1994). In the RXR subfamily, RXRα and β are widely distributed, whereas abundant expression of the γ subtype is limited to only a few tissues (Mangelsdorf and Evans, 1992; Dollè et al., 1994).

Of particular interest is the overlapping temporal and spatial expression of RARβ and RXRγ in several discrete areas of the developing central nervous system (CNS) (Giguère et al., 1987; Dollè et al., 1990, 1994; Ruberte et al., 1991; Mangelsdorf and Evans, 1992; Yamagata et al., 1994). While RAR and RXR heterodimers have been shown to be the functional units for transducing RA signaling during mouse embryogenesis (Kastner et al., 1997), coexpression of RARβ and RXRγ during brain development, as well as in the adult brain (Chiang and Evans, unpublished data), indicates that they contribute to specific functions in the CNS. As an approach to unmask these potential physiological roles, mice lacking either RXRγ or RARβ or both alleles were generated and examined for performance and learning deficits.

Although the neuromodulation of synaptic transmission by local signaling pathways is widely studied, much less is known about the control of synaptic properties through systems that involve nuclear transcription. Nonetheless, evidence suggests that nuclear events can play a role in the regulation of synaptic transmission. Many kinases either phosphorylate and activate latent cytoplasmic transcription factors, or in some cases they may go directly into the nucleus (for reviews, see Hunter and Karin, 1992; Karin and Hunter, 1995). Furthermore, even a "hardwired" adult brain can be dramatically influenced by steroid hormones, including estrogens, androgens, and glucocorticoids (Kandel et al., 1991). While neuronal effects of androgens have been well studied in songbirds (Lewicki and Konishi, 1995), little is known as to how steroids influence higher CNS functions in mammals. The expression of retinoid receptors in the adult CNS provided an opportunity to address this issue by examining the effects of mutations in cognitive functions, such as learning and memory.

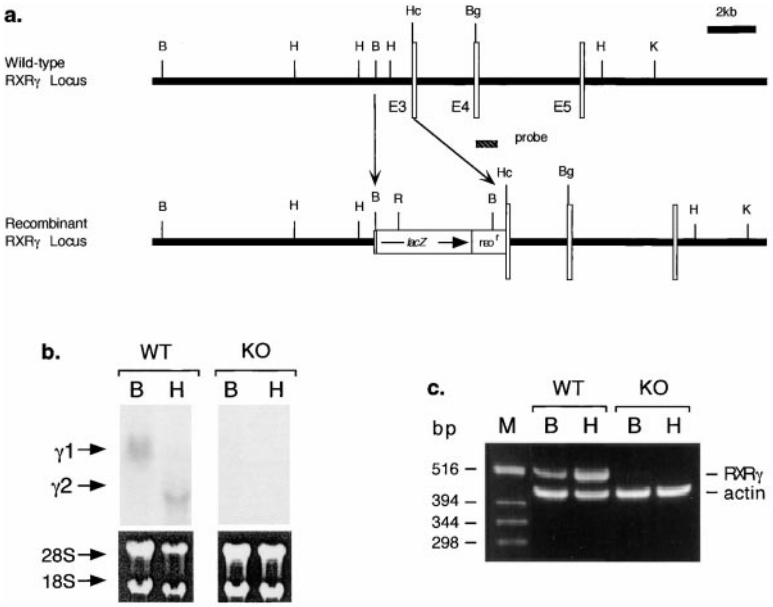

Figure 1. Targeted Disruption of the RXRγ Gene

(A) A schematic drawing of the RXRγ genomic region of interest is shown on top. The empty boxes indicate exons (E3, E4, and E5). The predicted structure of the recombinant mutant allele is shown at the bottom.

(B) Northern blot analysis of RXRγ transcripts in the wild-type and RXRγ mutant mice. Top, 10 μg of total RNA from the organs of adult mice of indicated genotype were blotted, immobilized, and probed with a full-length RXRγ cDNA. Both γ1 and γ2 transcripts are indicated. Bottom, ethidium bromide staining of the 28S and 18S ribosomal RNA in the gel.

(C) RT-PCR analysis of RXRγ transcripts in the wild-type and RXRγ mutant mice. 100 ng of total RNA and RXRγ- and actin-specific primers were used for each RT-PCR reactions. WT, wild-type; KO, knockout; B, brain; H, heart.

LTP and LTD are generally viewed as cellular mechanisms for learning and memory. They are activity-dependent long-lasting modifications of synaptic efficacy (Bliss and Collingridge, 1993). Hippocampal CA1 LTP and LTD are initiated postsynaptically by the activation of NMDA receptors, resulting in Ca^{2+} influx and the subsequent activation of several kinases (for reviews, see Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). While the involvement of Ca^{2+}-sensitive kinases in hippocampal synaptic plasticity has been confirmed by gene targeting studies (for review, see Chen and Tonegawa, 1997), their downstream substrates, including potential nuclear targets, are poorly understood.

In the case of RXRγ and RARβ/RXRγ genes, mutant mice are viable, fertile, show normal gross neural anatomy, and display no apparent abnormal behavior. Despite this apparent normality, further analyses of these mutants reveal profound performance deficits and strikingly altered neurophysiologic response. This is most dramatically manifested by nearly complete elimination of CA1 LTP in RARβ and RARβ/RXRγ double mutants. Surprisingly, the RXRγ knockout mice display a unique phenotype characterized by a complete but selective loss of LTD while their LTP remains unchanged. These findings uncouple CA1 LTD from LTP and suggest novel and specific roles for the vitamin A and its receptors in synaptic plasticity.

Results

Generation of RXRγ and RARβ/RXRγ Double Mutants

RXRγ knockout mice were created as previously described (Sucov et al., 1994). Homologous recombination of the targeting construct resulted in disruption of the entire RXRγ gene as assessed by Northern blot and RT-PCR analyses (Figure 1). RXR$\gamma^{-/-}$ mice were obtained at the expected Mendelian ratio and were indistinguishable from their wild-type or heterozygous littermates, an observation that is consistent with a previous report (Krezel et al., 1996).

RXRγ mutants were bred with RARβ knockout mice (Luo et al., 1995) to produce animals with null mutations in both genes. Interestingly, RARβ/RXRγ double null mutants were viable, fertile, and had no grossly discernible phenotype. Considering their prominent coexpression patterns in the adult brain (striatum, hippocampus, and cerebellum; data not shown), we began to search for more subtle phenotypes. As lesions in these areas often result in specific cognitive and motor deficits (O'Keefe and Nadel, 1978; Morris et al., 1982; Ivry and Baldo, 1992; Calabresi et al., 1996), we explored whether these receptors contribute to specific functions in the CNS. For simplicity, RXRγ and RARβ are designated hereafter as γ and β, respectively.

Mutant Animals Exhibit Performance Deficits in the Morris Water Maze

Mutant mice were first examined in the Morris water maze (MWM). MWM is a hippocampal-dependent test commonly used to evaluate the ability of rodents to solve a spatial learning task (Morris, 1984). During the acquisition phase (the hidden platform test), β and β/γ mutants displayed severely impaired abilities in accomplishing the task and failed to improve their performance over the training period (8 days), whereas γ mutants exhibited performances comparable to wild-type controls (Figure 2A). Swim speed was unaffected in all genotypes (data not shown). The abnormal behavior of the mutants was further exemplified by the probe trial in which the platform was removed. β and β/γ mutants spent significantly less time than wild-type animals in the target area where the platform had been (Figure 2B). The analysis of the mean distance to the target point also showed significant difference between both β and β/γ mutants and wild-type animals (Figure 2C). Although the performance of β and β/γ mutants was possibly compromised by a likely impaired vision (Grondona et

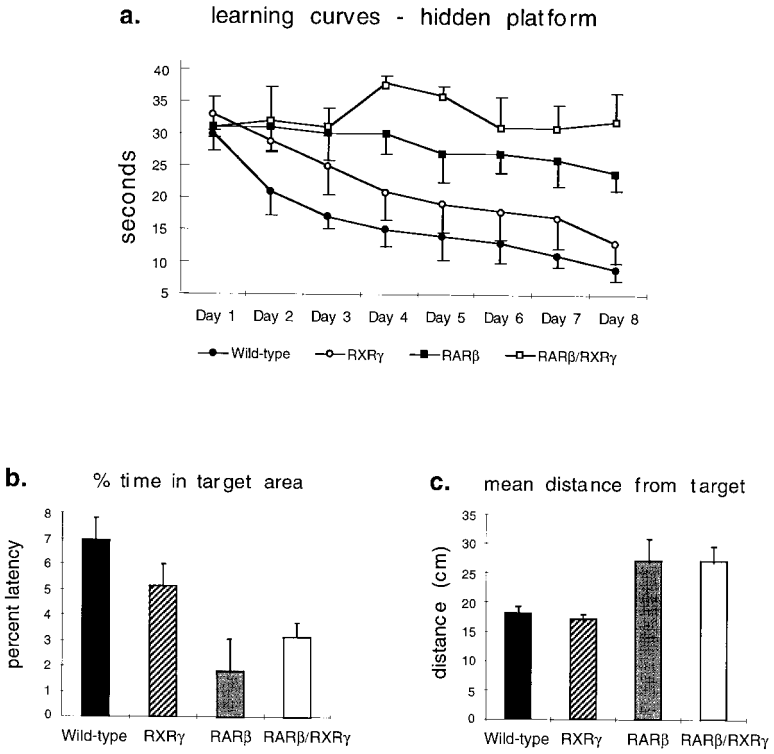

Figure 2. Performance Deficits of the Mutant Animals in the Morris Water Maze Tests

(A) The graph shows the escape latencies of mice trained to find a hidden platform in a water maze. Overall analysis revealed that wild-type mice needed 16.2 ± 1.2 sec to reach the platform, as opposed to 28.5 ± 1.2 sec for β, 32.6 ± 1.4 for β/γ, and 21.8 ± 1.5 sec for γ mutants. With p value of 0.0029 (ANOVA), the difference in performance was significant for wild-type versus β and β/γ mutants. No significant difference was found between β and β/γ double mutants. Note that on a day-by-day analysis, γ mutants consistently used more time to reach the platform than wild-type controls. However, the difference between the two groups was not statistically significant (ANOVA).

(B and C) The results of the probe trial given on day 9 of training. (B) The comparison of % latency at the target area. Both β and β/γ mutants spent significantly less time (β: 1.8 ± 1.0%; β/γ: 3.1 ± 0.9%) in the target area as compared to wild-type (6.9 ± 1.1%) or γ mutants (5.1 ± 1.3%). (C) The comparison of the mean distance to the target point. The mean distance analysis showed 18.0 ± 1.3 cm for wild-type, 17.1 ± 1.0 cm for γ, 26.9 ± 3.9 cm for β, and 27.0 ± 2.7 cm for β/γ mutants. Both wild-type and γ mutants were significantly different from β and β/r mutants ($p = 0.0115$, ANOVA). Data are expressed as mean ± standard error.

al., 1996), blind rats have been shown to improve their performance in this test (Lindner and Schallert, 1988; Lindner et al., 1997). Therefore, the observed poor performance of β and β/γ mutants in MWM could reflect an underlying problem in spatial learning and memory.

Mutant Animals Have Impaired Hippocampal CA1 LTP and LTD

As changes in the ability to modify hippocampal synaptic strength have been associated with learning impairment in rodents (for review, see Chen and Tonegawa, 1997), we carried out electrophysiological experiments to investigate the integrity of LTP and LTD in area CA1 of the hippocampus in the knockout mice.

A summary of LTP of normalized field excitatory postsynaptic potentials (fEPSPs) from slices of wild-type and mutants of all genotypes is shown in Figure 3. Surprisingly, we found that LTP induced by tetanic stimulation in β ($n = 17$) and β/γ ($n = 15$) mutant slices was essentially absent, with no significant difference in the magnitude between the two (Kolmogorov-Smirnov two-sample statistic). In contrast, LTP appeared intact in γ knockout slices, as their mean fEPSPs amplitudes were not statistically different from those of wild-type controls (Kolmogorov-Smirnov two-sample statistic; $n = 25$; Figures 3A and 3B).

As revealed in Figure 4A, low frequency stimulation (LFS) produced a significant long-lasting depression of the fEPSPs from wild-type slices ($n = 15$), whereas it induced only transient depression in all mutant samples ($n = 14$ for γ; $n = 13$ for β; $n = 13$ for β/γ), which returned to nearly baseline by 35 min following stimulation. When averaged across slices, LTD of all mutant slices was significantly different from controls (Kolmogorov-Smirnov two-sample statistic; Figures 4A and 4B).

We also determined the probability distributions of LTP and LTD amplitude for mutant and wild-type slices in the presence of 2-amino-5-phosphonopentanoate (AP5), an antagonist of N-methyl-D-aspartate (NMDA) receptors that disables LTP and LTD induction (Collingridge et al., 1983; Dudek and Bear, 1992). A shift of probability distribution from that of AP5 would indicate the presence of LTP or LTD. Figures 3B and 4B show the histograms of LTP and LTD, respectively, in which cumulative probability is plotted as a function of mean responses measured between 25 and 35 min after stimulation. Because the LTP probability distributions of β and β/γ slices are offset to slightly larger values than those of AP5 (statistically different based on Kolmogorov-Smirnov one-sample test; 0.05 significance level), LTP in these mutants is mostly but not completely absent (Figure 3B). Perhaps more dramatically, the mean LTD probabilities of all mutants center near 1.0 and are indistinguishable from the AP5 distribution, indicating that the mutant animals all lack LTD (Kolmogorov-Smirnov two-sample test; Figure 4B), at least with this standard induction protocol. Thus, β and β/γ mutants have greatly diminished LTP and virtually no LTD, whereas γ knockouts lack LTD but retain a normal LTP.

Depotentiation Is Absent in γ Mutants

To investigate further the apparent lack of LTD in γ mutants, depotentiation in these animals was investigated. Previous studies have demonstrated that depotentiation is consistently expressed in both young and old animals and can be induced in older slices in which

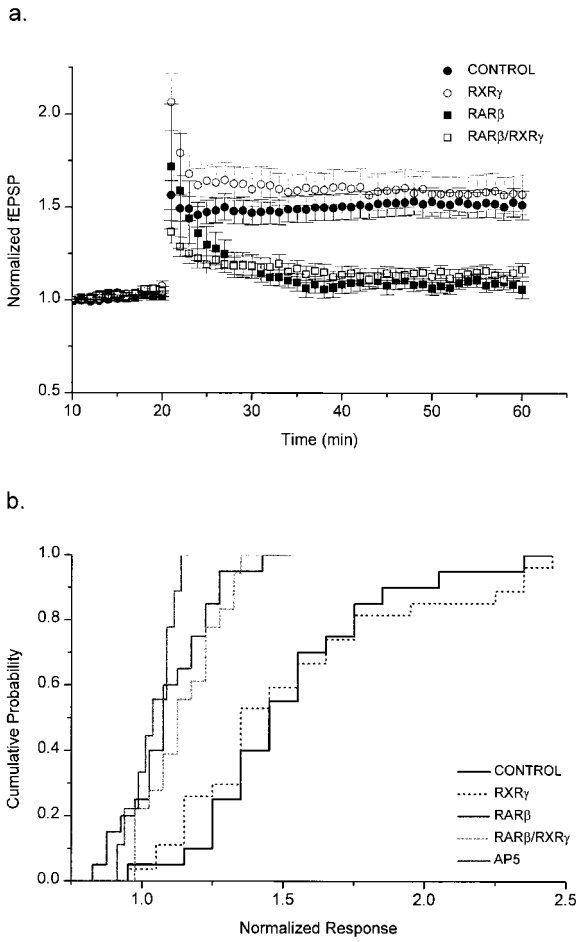

Figure 3. Hippocampal CA1 LTP

(A) Summary of field potential recordings from slices of control (closed circles; 1.517 ± 0.083; 17 slices, 6 mice), γ mutant (open circles; 1.576 ± 0.105; 25 slices, 11 mice), β mutant (closed squares; 1.098 ± 0.040; 17 slices, 6 mice), and β/γ mutant animals (open squares; 1.156 ± 0.040; 15 slices, 4 mice). The initial slope of fEPSPs is normalized to the baseline value preceding the induction of LTP. Data are expressed as mean ± SEM. Testing stimuli were given every 20 sec.

(B) Cumulative probability histogram of the magnitude of field potential LTP. Cumulative probability is shown as a function of mean response 25–35 min after tetanic stimulation. AP5 data are included (10 slices, 2 mice).

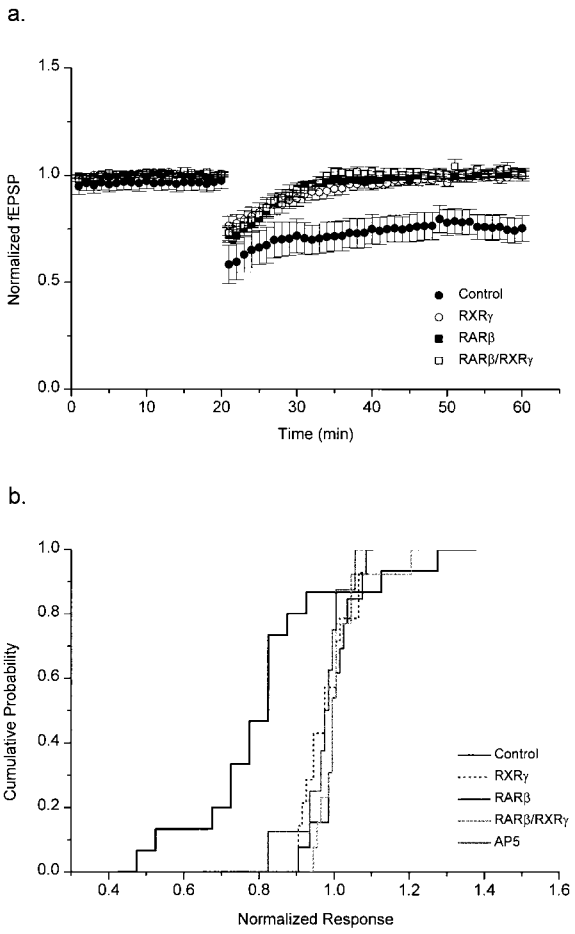

Figure 4. Hippocampal CA1 LTD

(A) Summary of field potential recordings from slices of control (closed circles; 0.771 ± 0.061; 15 slices, 6 mice), γ mutant (open circles; 0.983 ± 0.018; 14 slices; 4 mice), β mutant (closed squares; 0.994 ± 0.014; 13 slices; 4 mice), and β/γ mutant animals (open squares; 1.011 ± 0.019; 13 slices; 5 mice). The initial slope of fEPSPs is normalized to the baseline value preceding the induction of LTD. Data are expressed as mean ± SEM. Testing stimuli were given every 20 sec.

(B) Cumulative probability histogram of the magnitude of LTD. Cumulative probability is shown as a function of mean response 25–35 min after low frequency stimulation. AP5 data are included (8 slices, 2 mice).

LTD was not previously induced (Wagner and Alger, 1995). As synaptic activity preceding LFS has been shown to increase the probability of subsequent induction of LTD (Christie and Abraham, 1992; Wexler and Stanton, 1993; Wagner and Alger, 1995), we primed the slices with tetanic stimulation (induction of LTP) before the attempt to induce LTD. Slices in which robust LTP was observed were then stimulated at a low frequency (1 Hz) for 15 min. While the slices initially responded to the stimulation, the response quickly returned to baseline values, and no appreciable LTD was observed ($n = 5$; Figure 5). These experiments further demonstrated that LTD in γ mutants could not be induced.

Mutant Synapses Are Structurally and Functionally Normal

To examine the synapses of the mutant animals in more detail, electron-microscopy analysis was carried out. Importantly, the mutant animals show no apparent differences in their ultrastructure relative to the control animals (Figure 6). The synaptic junctions in CA1, like other brain regions, are defined by electron dense postsynaptic densities and active zones (see, for example, Schikorski and Stevens, 1997). Like control animals, mutant excitatory synapses in the CA1 region of the hippocampus are mainly located on spines. Their presynaptic boutons are filled with synaptic vesicles and a few vesicles are docked at active zones.

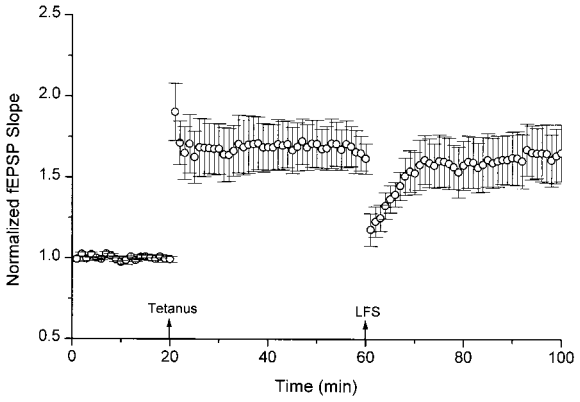

Figure 5. Depotentiation Is Absent in γ Mutants

Summary of field potential recordings following tetanus and low frequency stimulation (LFS) (n = 5). The initial slope of fEPSPs is normalized to the baseline value proceeding the induction of LTP. Data are expressed as mean ± SEM.

We further used whole-cell patch clamp techniques to investigate synaptic transmission of the mutant animals. The amplitude and time course of synaptic currents at various voltages appeared normal in mutant neurons (data not shown). Changes in the postsynaptic responsiveness to glutamate are reflected in the amplitudes of spontaneous miniature excitatory postsynaptic currents (mEPSCs), while changes in the presynaptic release process are indicated by the frequency of mEPSCs (Thompson et al., 1993). The mEPSCs of both wild-type and mutant neurons showed similar amplitude distributions and frequencies, with no statistical difference between the two animal groups (Kolmogorov-Smirnov two-sample statistic; Figures 7A and 7B), indicating that presynaptic release and postsynaptic response to glutamate were unimpaired in the mutants.

We also employed paired-pulse facilitation (PPF) to test for abnormal presynaptic neurotransmission. PPF is an enhancement of the synaptic response to the second of two closely spaced stimuli and is increased if the probability of neurotransmitter released presynaptically is lowered (Creager et al., 1980; Zucker, 1989; Manabe et al., 1993; Thompson et al., 1993; Mennerick and Zorumski, 1995; Debanne et al., 1996). Over an interpulse interval range of 20–200 ms, the mutant and wild-type neurons are virtually identical (Kolmogorov-Smirnov two-sample statistic; Figure 7C). Thus, presynaptic neurotransmission appears normal in mutant slices.

Finally, since NMDA receptor activation is required for both LTP and LTD (Dudek and Bear, 1992; Collingridge et al., 1983), we examined the functionality of NMDA receptors by monitoring EPSCs of the same cell before and after application of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist of non-NMDA receptors. The time course and magnitude of the NMDA receptor components did not differ between wild-type and mutant cells (data not shown), nor did the ratios of NMDA receptor component to total synaptic currents (control: 0.103 ± 0.035, n = 4; β/γ mutants: 0.101 ± 0.023, n = 6; $p < 0.05$; two-sample t-test). These data reveal the presence and functional equivalence of NMDA receptors in both control and mutant animals.

Taken together, these results establish that the hippocampal CA1 synapses are both morphologically and functionally normal in the mutant animals. Thus, the observed deficiency in LTP and/or LTD probably reflects deficits in the mechanisms responsible for hippocampal synaptic plasticity.

Discussion

In this manuscript, we provide unexpected evidence of a role for receptors of vitamin A and its metabolites in the regulation of synaptic plasticity. Although retinoids are essential for normal CNS development, their known roles are mostly restricted to well-characterized gross developmental defects, none of which are seen in β, γ, or β/γ mutants. In contrast, little is known regarding specific functions of retinoids even though receptors continue to be expressed in the adult CNS. Here, we demonstrate that mice deficient in RARβ, though viable, fertile, and apparently normal, lack CA1 LTP and LTD and exhibit profound performance deficits in spatial learning and memory tasks. Surprisingly, RXRγ appears to be required for CA1 LTD yet animals deficient in this function are only mildly compromised in maze learning. Our discoveries establish unexpected links between retinoids and normal hippocampal functions and add new concerns to the health consequences of vitamin A deficiency.

Analyses of null mice lacking Ca^{2+}-sensitive kinases

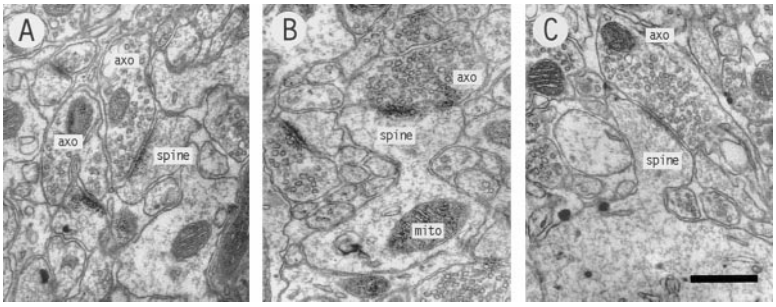

Figure 6. Mutant Synapses Are Structurally Normal

Ultrastructure of synapses in CA1 region of hippocampus of a wild-type (A) RARβ (B) and RXRγ (C). In all animals, spines (spine) form asymmetric contacts and are apposed by axonal boutons (axo). Synaptic junctions are defined by postsynaptic densities and active zones. The bouton is filled with synaptic vesicles and a few vesicles are docked at the active zone. Mitochondria (mito) are present within the dendritic shafts and the axonal boutons but are missing within the spine head. Scale bar: 0.5 μm for all three images.

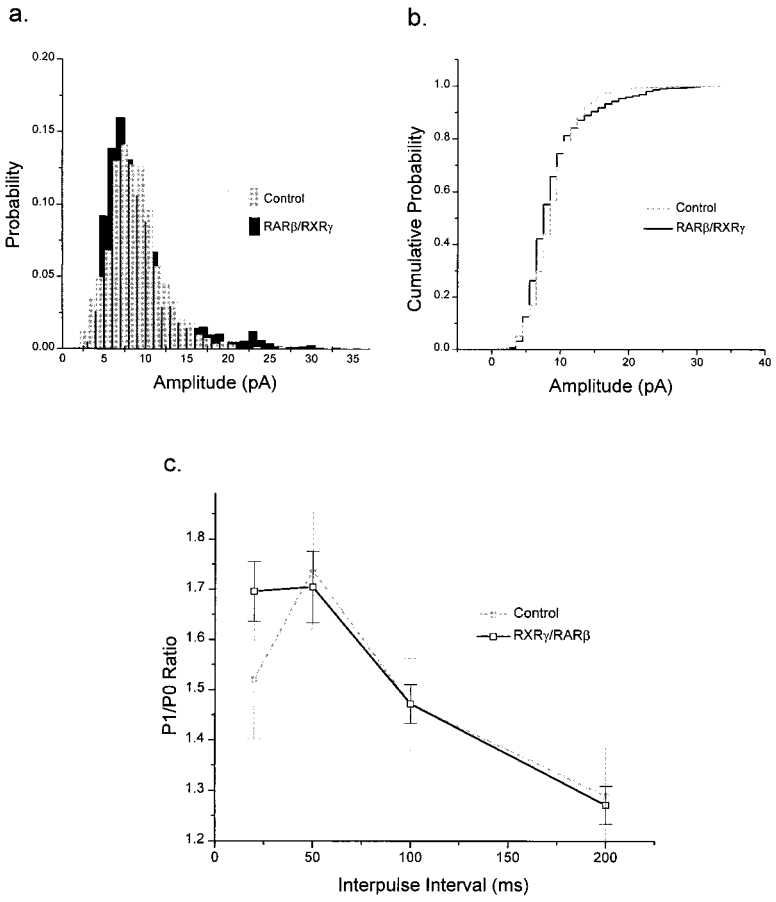

Figure 7. Synaptic Transmission Appears Normal in Mutant Animals

(A) Amplitude histogram of spontaneous miniature EPSCs. The extracellular solution contained 50 mM sucrose and 1 μM tetrodotoxin for these recordings. The threshold for peak detection was set between 5 and 7 pA. Data were binned in 1 pA intervals. The mean frequency and mean amplitude of mini EPSCs were 1.178 ± 0.128/sec and 8.848 ± 0.888 pA for mutant cells (n = 6) and 1.125 ± 0.16/sec and 8.753 ± 0.881 pA for control cells (n = 6). (B) Cumulative probability histogram of the amplitudes of miniature EPSCs. (C) Paired-pulse facilitation. The plot shows the summarized (mutant: 7 cells; wild-type: 8 cells) facilitation of the second EPSC (P1) compared with the first EPSC (P0) as a function of the interpulse interval. Holding potential was −70 mV. Error bars show SEM.

(Abeliovich et al., 1993a, 1993b; Silva et al., 1992a, 1992b) or components of the cAMP pathway (Bourtchouladze et al., 1994; Wu et al., 1995; Kogan et al., 1997), overall, have correlated impairments in synaptic plasticity and certain types of learning. Our results from the combined electrophysiological and behavioral studies are consistent with the hypothesis that CA1 LTP underlies spatial learning and memory. The fact that many kinases translocate into the nucleus upon activation (for reviews, see Hunter and Karin, 1992; Karin and Hunter, 1995) has long been suggestive of a nuclear function in some sort of synaptic plasticity. However, the identity and function of such targets has remained obscure.

How might retinoid receptors, or transcription factors in general, contribute to synaptic plasticity, which in the context of the initiating event is unlikely to depend on protein synthesis, much less transcription? In one view, the defect is developmental, leading to altered hippocampal morphology and function. However, the presence of normal synaptic morphology and NMDA receptor function in the mutants suggests this explanation to be unlikely. Furthermore, in the RXRγ mutants LTP is normal, indicating the key circuitry is intact. Second, perhaps more likely, one or more critical components of the signal transduction pathway are retinoid dependent and therefore fail to be produced in the mutant animals. In this view, neither LTP nor LTD is required for normal hippocampal development. Third, but less likely, the receptor performs a required and direct role in each potentiation event. While the RAR is the first transcription factor shown to be essential for LTP and LTD, mutations in the CREB gene clearly reduce the magnitude of CA1 LTP (Bourtchouladze et al., 1994). CREB is a transcription factor implicated in the regulation of adaptive neuronal responses. Following membrane depolarization, it becomes phosphorylated and activates the expression of downstream genes whose protein products function at synapses to control neuronal activities. One of such target genes is the brain-derived neurotrophic factor (BDNF) (Shieh et al., 1998; Tao et al., 1998). Synaptic transmission and LTP are severely impaired in the hippocampus of BDNF knockout mice and can be rescued by viral expression of BDNF slices or by bath application of BDNF (Korte et al., 1995, 1996; Patterson et al., 1996). Interestingly, the defects are also seen in heterozygous animals, indicating that a critical level of BDNF may be required for LTP. Since there are multiple BDNF promoters (Timmusk et al., 1993), it is conceivable that other transcription factors could affect its expression. While we did not find consensus retinoid response elements in the reported promoter sequences of the BDNF gene, this serves as an illustration that functions requiring gene expression and the activation of downstream targets of the RAR/RXR heterodimers most likely lie at the heart of the CNS dysfunction.

The lack of both CA1 LTP and LTD in mice deficient in RARβ implies that these two forms of plasticity are

dependent, directly or indirectly, on retinoids. It also suggests that RARβ is a crucial component in transducing retinoid signaling in the hippocampus. The finding that γ knockouts lack LTD but retain LTP indicates that at least a second RXR family member is able to substitute as an RARβ partner in LTP. Neural expression of RXRβ suggests this as a likely candidate (Mangelsdorf et al., 1992; Krezel et al., 1998). However, in view of the spatial and temporal coexpression of both the RARβ and RXRγ genes in several discrete areas in the embryonic and adult central neurons, RARβ/RXRγ heterodimers are apparently a critical functional unit.

In addition to the spatial learning deficits, we also found β and β/γ knockouts are severely impaired in motor coordination and balance (rotarod test, data not shown), an observation consistent with a recent report (Krezel et al., 1998). Histological examination of the mutant brains did not reveal any overt structural abnormalities in any regions where RARβ and RXRγ are abundantly expressed (data not shown); neither did we detect any significant alterations in the expression of several neuropeptides, such as glutamic acid decarboxylase (GAD)-67, enkephalin, substance P, and dynorphin (data not shown). This study suggests that lack of retinoid signaling does not significantly affect neuronal development. Additional detailed examination of regional brain morphology in conjunction with additional functional tests is required to better understand the neuronal circuitry involved in the deficits exhibited by these mutant mice.

Since its discovery in 1915, Vitamin A has been known to be necessary for normal growth and vitality (Sporn et al., 1994). VAD affects nearly 190 million children worldwide, all of whom are at high risk for growth defects (72%), xeropthalmia (11%), and numerous health-related problems. Severe deficiency (indicated by childhood blindness) occurs in approximately 500,000 new cases annually, with half of these cases dying within one year (Sommer, 1982). Severe visual problems in conjunction with extreme poverty and the field conditions associated with VAD has made it difficult to provide any clear assessment of the role of Vitamin A in higher cognitive functions, including learning and memory. Our discovery of the role of retinoid receptors in higher brain functions in rodents adds additional concerns to the health consequences of VAD and suggests that its roles in human CNS function should be reevaluated.

Experimental Procedures

Targeting Construct

The 1.6 kb BamHI/HincII genomic fragment was replaced with a β-gal/PGK-neo cassette. In this configuration, the 3′ end of the intron immediately upstream of exon 3, the splicing acceptor, and part of exon 3 were deleted. A 180 bp fragment generated by PCR using primers flanking the intron/exon 3 junction was introduced back into the targeting construct. The PCR primers used were 5′-CTGTGGATCCAACATTTAGCTGCAAGAG-3′ and 5′-TGACAGATCT TCATGTCATAGATGAGCAG-3′. A herpes simplex virus-thymidine kinase (HSV-TK) cassette was included at the 3′ end of the construct.

ES Cell Culture, Gene Targeting, and Generation of Knockout Mice

ES cells of the J1 line (Li et al., 1993) were grown on a feeder layer of G418 resistant embryonic fibroblasts. Not I-linearized targeting construct was electroporated into ES cells using a Bio-Rad gene pulser under conditions described previously (Li et al., 1993). Cells were selected with 175 μg/ml G418 and 0.2 μM FIAU; individual colonies were picked after 8–10 days of selection and expanded for DNA preparation. Southern blot analysis was used to identify cells that have undergone homologous recombination. ES cells that contained a disrupted RXRγ allele were then introduced into C57BL/6 blastocysts by microinjection to generate chimeric mice. Chimeras transmitting mutant alleles through the germline were crossed with C57BL/6 mice to generate heterozygous animals, which then were intercrossed to obtain knockout mice. All mutants were raised on a mixed genetic background of 129/Sv and C56BL/6.

Northern Blot Analysis

Total RNA was isolated from various organs of wild-type and RXRγ$^{-/-}$ mice by single step isolation using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH). 20 μg of total RNA per lane was separated on 1% agarose-formaldehyde gel, transferred to nitrocellulose membrane in 20× SSC, and fixed by baking at 80 °C for 2 hr. The 1 kb EcoRI/ScaI mRXRγ cDNA fragment, containing the hinge region and ligand binding domain, was labeled with αP^{32}-dCTP and hybridized to the filters. Hybridization was carried out at 68°C for 1 hr in QuickHyb hybridization solution (Stratagene, La Jolla, CA), followed by two washes in 2× SSC/0.1% SDS at RT and two washes in 0.2× SSC/0.1% SDS at 60°C. The blots were exposed to Biomax film (Kodak) overnight with an intensifying screen at −70°C.

RT-PCR Analysis

100 ng total RNA from various tissues was used in the RT-PCR reactions. RXRγ-specific (5′-TCTGGTGAAACACATCTGTGCC-3′ and 5′-AGAATGACCTGGTCCTCCAAGG-3′; these are primers common to both isoforms, γ1 and γ2) and actin-specific (5′-CGGAGGGA AAGATTCCTCTGCC-3′ and 5′-AGGGCCGGCACATTGAAGGTCT-3′) primer sets were included in each reaction, which gave rise to a 500 bp and a 430 bp band, respectively. The Titan RT-PCR kit (Boehringer Mannheim) was employed. Reactions were set up following the manufacturer's instructions and carried out in an MJR thermocycler.

Morris Water Maze Tests

Four groups of seven 3-month-old male animals (control, RARβ, RXRγ, and RARβ/RXRγ mutant mice) were included in this study. Control animals are wild-type littermates of RARβ knockout mice. Mice were placed in a round pool filled with water made opaque with non-toxic paint. To escape from the water, the animals had to locate a platform submerged 1 cm below the water surface (the hidden platform test). Data, such as the time the animals took to reach the platform (latency) and the length of the swim path, were recorded with an automated video tracking system (San Diego Instruments). For an overall analysis (performance over days), the mean value per day was calculated and analyzed with a two-way ANOVA and Fisher post-hoc test. For a day-by-day analysis (performance over trials), the values for each trial were analyzed in a two-way ANOVA.

In the hidden platform test, each mouse was placed into the water at a different position in each trial; each trial lasted 40 sec or until the animal found the platform. All animals were allowed to stay on the platform for 10 sec between trials. Four trials were tested per day on eight consecutive days. On the ninth day, the platform was removed and the mice were allowed to search for the platform for 40 sec (probe trial).

Electrophysiology

LTP experiments were performed on mice between 1 and 13 months of age. No significant difference was observed between younger and older mice. LTD experiments were done on mice 2 to 4 months of age. Whole cell experiments were performed on 13- to 16-day-old mice. Control animals are wild-type littermates of RARβ knockout mice.

Mice were anesthetized by Metofane and decapitated. The brain was removed and placed in ice-cold solution (120 mM NaCl, 3.5 mM KCl, 0.7 mM $CaCl_2$, 4 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM

$NaHCO_3$, and 10 mM glucose) bubbled with 95% O_2/5% CO_2. The same cold high Mg^{++}, low Ca^{++} solution was used throughout the dissection procedure to prevent transmitter release and minimize injury to the cells. The hindbrain was cut away, and the flat surface of the forebrain was glued to the pan of a DSK Microslicer vibratome with cyanoacrylate glue. The hippocampus of each 350 μm slice was dissected out and placed in a chamber containing the same dissecting buffer perfused with 95% O_2/5% CO_2. Slices were allowed to incubate at room temperature in the holding chamber for at least 1 hr prior to recording. All experiments were performed at room temperature. Individual slices were placed in a submerged recording chamber, held by a net made with flattened platinum wire and nylon threads. Slices were perfused with solution (120 mM NaCl, 3.5 mM KCl, 2.6 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose) saturated with 95% O_2/5% CO_2, at a rate of 2 ml/min. A cut was generally made between the CA1 and CA3 region to prevent recurrent excitation. Schaeffer collateral-commissural fibers were stimulated by ultrasmall concentric bipolar electrodes (FHC Inc.) delivering 0.1 ms pulses. Field potentials were recorded in field CA1 with glass electrodes (2–3 Mω resistance) filled with perfusing buffer. Whole-cell patch clamp recordings were performed in CA1 pyramidal neurons according to standard techniques. The pipettes (3.5–5 M resistance) were filled with a solution containing 170 mM K-Gluconate, 10 mM HEPES, 10 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 3.5 mM Mg-ATP, 1.0 mM GTP-Li (pH adjusted to 7.2), and 292 mOSM. Stimulus intensity was adjusted to evoke similarly sized baseline responses. LTP was induced by a tetanus consisting of five trains of 100 Hz stimulation lasting 200 ms at an intertrain interval of 10 sec. LTD was evoked by 900 stimuli at a frequency of 1 Hz. In whole-cell recordings, the membrane potential of the postsynaptic cell was held at −70 mV, except during NMDA experiments, at which time they were held at −50 mV. Recordings were performed using an Axopatch 200 amplifier, filtered at 2 kHz, and analyzed with programs written in Visual Basic. The initial slopes of fEPSPs and peak amplitudes of EPSCs were measured for field potential and whole-cell recordings, respectively. AP5 and tetrodotoxin were obtained from Research Biochemicals Inc. and CNQX from Cambridge Research Biochemicals. Data are expressed as mean ± SEM.

Electron-Microscopy Analysis

Mice (control, RARβ mutant, and RXRγ mutant; n = 1) were perfused through the heart under deep Nembutal anesthesia (0.1 ml/animal). A short perfusion of oxygenated saline containing 0.5 μl TTX (Sigma) was followed by a perfusion with 1.6% paraformaldehyde, 1.6% glutaraldehyde, and 0.4% acrolein in s-Collidine buffer (all by Electron Microscopy Sciences, EMS) (1 mM $CaCl_2$ added) at room temperature (RT). Brains were dissected and immersed in the same fixative at 4°C for 3 hr. Vibratome sections (300 μm) were cut in HEPES-buffered saline (20 mM HEPES, 145 mM NaCl, and 2 mM $CaCl_2$) at 4°C. Sections were fixed in 0.2% tannic acid (EMS), washed, and transferred to s-Collidine buffer and post fixed in 1% OsO_4 and 1.5% K- ferrocyanide at 4°C for 1 hr. After washing in distilled water, the sections were contrasted en bloc in 1% uranyl acetate (EMS) at RT for 1 hr. Tissue blocks containing the hippocampus were dehydrated in ethanol and embedded in Epon. Embedded tissue blocks were trimmed to small blocks including only the CA1 region and cut ultrathin at silver. Sections were collected on formvar grids and stained with lead citrate. Stained sections were inspected and photographed by using a Joel 100 CX II electron microscope.

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